Kinetic studies on thermal denaturation of C-phycocyanin

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The kinetics of thermal denaturation of a biliprotein, C-phycocyanin (C-PC) isolated from Spirulina platensis were studied at different pH values, ranging from 4.0 to 8.0. The denaturation of C-PC follows the first order kinetics and rate constant at pH 5.0 and temperature 55ºC is found to be $4.37 \times 10^{-5}$ s$^{-1}$, which increases to $5.46 \times 10^{-5}$ s$^{-1}$ at pH 7.0. The denaturation rate is much higher at 65ºC and pH 7.0 ($7.96 \times 10^{-4}$), as compared to at pH 5.0 ($1.46 \times 10^{-4}$). The thermal stability of C-PC is more at pH 5.0, as compared to other pH values. The observed differences in entropy values at pH 5.0, as compared to other pH values indicate a considerably close fit structure of the protein at pH 5.0, which increases the stability of native structure, even at higher temperature (65ºC).

Keywords: Phycobiliproteins, Spirulina platensis, C-Phycocyanin, thermal denaturation, first order kinetics.


Phycobiliproteins, the metal free tetrapyrrole pigment proteins play a vital role in photosynthesis in certain microalgae, including cyanobacteria and red algae. They are broadly categorized into three groups, based on their spectroscopic properties — phycocerythrin ($\lambda_{\text{max}}$, 540-570 nm), C-phycocyanin (C-PC) ($\lambda_{\text{max}}$, 615-620 nm) and allophycocyanin (APC, $\lambda_{\text{max}}$, 650-655 nm). They form highly ordered macromolecular assemblies known as phycobilisomes in vivo, which play a role in light harvesting and energy migration, usually to photosystem-II (PS II) reaction center. In addition, their unique physical and spectroscopic properties are receiving increasing attention, in view of wide applications. Their fluorescent nature makes them suitable for highly sensitive fluorescence-based applications, such as flow-cytometry, fluorescence-activated cell sorting, fluorescence immunoassay and fluorescence microscopy$^{1,2}$.

The nutritional and therapeutic values of Spirulina platensis are well documented$^{3,4}$. C-PC is an important constituent of S. platensis and has assumed importance for its promising antioxidant, anti-inflammatory and anticancer properties due to its radical scavenging activity$^{5,7}$. In view of the increasing applications of C-PC in the field of biomedicals, in the present paper, the denaturation kinetics of C-PC, as a function of temperature at different pH values is reported.

Materials and Methods

DEAE Sepharose CL-6B was obtained from Sigma Chemical Co, St. Louis, MO, USA. All other reagents used were of A.R. grade available from commercial sources.

C-PC purification

Fresh water cyanobacterium S. platensis was grown in batch cultures in Zarrouk’s medium$^8$ at pH 10, temperature 20±2°C and optimum light intensity of 60 µ E m$^{-2}$ s$^{-1}$, provided by cool-white fluorescent tubes with a dark:light cycle of 12:12 hr. Fresh cells were harvested after 15 days of incubation by centrifugation at 10000 $\times$ g for 30 min and cell mass was washed twice with distilled water and freeze dried. The known weight of freeze dried cell mass was suspended in Na-phosphate buffer (0.1 M, pH 7.0 containing 1 mM sodium azide) and disrupted by sonication for 60 sec. Repeated freezing at –20°C and thawing at room temperature in dark, followed by centrifugation at 10000 $\times$ g for 30 min at 4°C gave a clear supernatant containing C-PC and APC.

All purification steps were carried out in dark at 10-15°C and all the buffers used contained 1 mM sodium azide, unless stated otherwise. The crude extract of C-PC was fractionated with solid (NH$_4$)$_2$SO$_4$ at 25 and 50% saturation, respectively and the precipitate from 25% saturation was discarded. The supernatant was further brought to 50% saturation with solid (NH$_4$)$_2$SO$_4$ and allowed to stand for 4 hr at 4°C. The precipitated protein mass contained mainly C-PC, which was collected by centrifugation at 10000 $\times$ g for 30 min at 4°C, resuspended in acetate buffer (0.1 M, pH 4.5) and centrifuged again at 10000 $\times$ g for 30 min at 4°C. The
precipitate was discarded and the supernatant again brought to 50% saturation with solid (NH₄)₂SO₄ and allowed to stand for 4 hr at 4°C, prior to centrifugation at 10000 × g for 30 min at 4°C. The precipitated C-PC was dissolved in minimum volume of Na-phosphate buffer (0.005 M, pH 7.0) and dialyzed overnight at 4°C against same buffer.

The dialyzed solution of C-PC was chromatographed on a DEAE-Sepharose CL-6B column (1.5 × 15 cm) and the column was developed with a linearly increasing ionic concentration gradient of NaCl solution (0-0.25 M) at flow rate of 0.5 ml/min. The C-PC was eluted between 0.15-0.25 M NaCl concentrations and was collected in 2 ml fractions. The purity of fractions was monitored by recording the absorption spectrum from 250 nm to 800 nm and the fractions having purity ratio recording the absorption spectrum from 250 nm to 800 nm and the fractions having purity ratio.

Thermal denaturation study

The thermal denaturation kinetics was studied at different temperatures (45°C, 55°C and 65°C), and denaturation of C-PC (0.33 mg/ml) was monitored spectrophotometrically by recording absorbance at 615-620 nm (λ_max) with Varian EL 01075086 spectrophotometer with temperature-control device. The absorbance was recorded after every 5 min interval for 60 min for kinetic study.

To evaluate the effect of pH on thermal stability of C-PC, the experiment was carried out at different pH values ranging from 4.0 to 8.0. Na-phosphate buffer (0.05 M) was used for pH control in the range 6.0 to 8.0, whereas Na-acetate buffer (0.1 M) was used for pH range 4.0 to 5.0.

Thermal denaturation of C-PC at different pH values follows the first order kinetics and the rate constant for the reaction was calculated according to the equation:

$$k_o = 1/t \ln \frac{n_1}{n_2}$$  \hspace{1cm} (1)

where \(k_o\) is the rate constant, \(t\) is time and \(n_1, n_2\) are native and denatured proteins, respectively. Thermodynamic parameters i.e., heat of activation and entropy of activation were calculated according to the following equations:

$$\Delta H = \text{Ea} - RT$$  \hspace{1cm} (2)

$$\Delta S = \log \frac{k}{T} + \frac{\Delta H}{2.3RT} - \log K/h$$  \hspace{1cm} (3)

where \(\Delta H\) is heat of activation, \(\text{Ea}\) the activation energy, \(R\) the gas constant, \(T\) the temperature, \(\Delta S\) the entropy of activation, \(k\) the rate constant, \(K\) the Boltzmann constant and \(h\) is Planck constant.

The activation energy \(\text{Ea}\) was calculated using the following equation:

$$\text{Ea} = R(T_1T_2/T_2 - T_1) \ln (k_2/k_1)$$  \hspace{1cm} (4)

where \(k_1/k_2\) is the ratio of rate constants at two temperatures \(T_1\) and \(T_2\).

Results and Discussion

The native or functional structure of a protein is a minimum energy state that is held together by an intricate balance of a multitude of covalent (ion dipole and hydrogen bonds) and non-covalent interactions (hydrophobic and vander Waal interactions). Thermal denaturation causes a combination of coulombic repulsion and thermal vibration leading to a non-proteolytic modification of the unique structure in the native protein, giving rise to definite changes in chemical, physical and biological properties.

Thermal denaturation of C-PC at different pH follows the first order kinetics. The rate constants \((k)\) for the reaction calculated at different temperatures under different conditions of pH are shown in Table 1. The value of \(k\) increases significantly when the temperature is increased from 45°C to 65°C, at all the pH values. The rate of thermal denaturation is almost similar for pH 5.0, 6.0 and 7.0 at 45°C and 55°C (Fig.1A and B) indicating that C-PC is quite stable at 45°C and 55°C at pH 7.0, 6.0 and 5.0. However, an enormous difference is observed between pH-5.0 and other pH values (4.0, 6.0, 7.0 and 8.0) at 65°C (Fig. 2C). The maximum stability is observed at pH-5.0.

Moreover, the thermodynamic data of thermal denaturation in C-PC at pH 4.0 and 5.0 differ from that at pH 6.0, 7.0 and 8.0 in the lower value of \(\Delta H\) and negative value of \(\Delta S\) (Table 1), indicating the maximum stability at 65°C and pH 5.0, due to lower \(\Delta S\) value. The higher negative value of \(\Delta S\) indicates highly associated form of C-PC and its close fit structure between protein and the rigid chromophore group (tetrapyrrrole ring) at pH 5.0. The negative values of \(\Delta S\) at pH 4.0, 5.0 (Table 1) may be due to the immobilization of water molecules by the activated complex at these pH values whereas the positive values at pH 6.0, 7.0 and 8.0 indicate that this phenomena is reversed at pH-6.0, 7.0 and 8.0.
Fig. 1—Denaturation of C-PC at different temperatures (A): at 45°C; (B): at 55°C; and (C): at 65°C

Table 1—Thermal denaturation of C-PC

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp. (°C)</th>
<th>$k_a$ (sec$^{-1}$)</th>
<th>$\Delta H$ (kcal/mole)</th>
<th>$\Delta S$ (e.u.)</th>
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</tr>
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<td>65</td>
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</table>

$k_a$, first order rate constant; $\Delta H$, free energy of activation; and $\Delta S$, entropy of activation

Fig. 2—Absorption spectra of C-PC at different pH values (A): at pH 4.0; (B): at pH 5.0; (C): at pH 6.0; (D): at pH 7.0; and (E): at pH 8.0
The UV-vis absorption spectra examined as a function of temperature at various pH values (4.0 to 8.0) show a major change in absorbance values at 65°C, due to the change from the native to denatured state (Fig. 2 A-E). Furthermore, the absorption maximum shifts towards the blue region with increasing temperature at all the tested pH conditions (Fig. 2 A-E). At pH 8.0, the absorption maximum shows ~11 nm blue shift, whereas at other pH values show ~2-3 nm blue shifts. The possible reason for the blue shift could be the conformational changes of terapyrrole chromophores, due to the unfolding of protein part, which imposes a particular conformation to chromophore in native C-PC structure.

Thus, the study presents data on thermal stability of C-PC under various conditions of pH. The C-PC denatures more rapidly above 45°C at different pH and shows unusual stability, even at higher temperature at particular pH (5.0).

References